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Phosphatase Activity Is a Constant Feature of All Isolates of All Major Species of the Family *Enterobacteriaceae*

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In this study we evaluated phosphatase activity in members of the family *Enterobacteriaceae* by conventional methods and by a novel method. The novel method is based on the formation of bright-green-stained colonies by phosphatase-positive, but not phosphatase-negative, strains in the presence of a phosphate substrate, such as phenolphthalein monophosphate or 6-benzoylnaphthyl phosphate (6-BNP), and methyl green. A total of 1,055 strains belonging to 65 different species of *Enterobacteriaceae* were tested for green staining of the colonies in the presence of methyl green and either phenolphthalein monophosphate or 6-BNP and for phosphatase activity by three different conventional methods. With the sole exception of one *Leminorella richardii* type strain, all isolates of all of the species formed green-stained colonies in the presence of the substrate 6-BNP. All strains were phosphatase positive by all of the conventional methods.

Evaluation of phosphatase activity has been profitably used in bacterial taxonomy (18, 21, 37), as well as for separation of pathogenic strains from similar nonpathogenic species (1, 3, 11, 12, 16, 20, 25, 31, 36).

By contrast with most bacterial groups of importance for human disease, in the family *Enterobacteriaceae* the evaluation of phosphatase activity has hitherto proved neither sufficiently extensive nor exhaustive. The pioneering studies often included *Enterobacteriaceae* among the strains considered (7, 24, 35), but with only one exception (35) none of them evaluated the incidence of phosphatase activity among the different species of *Enterobacteriaceae*. In the study in which this incidence was evaluated, the researchers concluded that phosphatase was frequently (though not always) produced by isolates of the *Proteeae* tribe but virtually never by any isolate belonging to any of the other species considered (35). Further studies which evaluated phosphatase activity in strains of several species of *Enterobacteriaceae*, as well as in other bacterial families, yielded data that sharply contrasted with one another and with those presented in the aforementioned study. Moreover, all of these studies considered very limited numbers of species or isolates of *Enterobacteriaceae*. In a study by Rao and Shivanada (26) five of eight isolates identified as *Proteus* spp. were found to be phosphatase negative, while both *Klebsiella* spp. and *Citrobacter* spp. were found more often than not to be phosphatase positive; no phosphatase activity was found among the *Escherichia coli* isolates. On the other hand, Tonelli (33), Cocks and Wilson (9), and Bray and King (7) found all strains of all of the species tested to be endowed with phosphatase activity, but their reports dealt with few species and very few strains. Finally, Kaluzewski (19) reported that the phosphatase reaction was positive after 24 h of incubation for both *Proteus vulgaris* and *P. mirabilis*, but it turned positive only after 2 to 5 days for *Escherichia coli* and *Salmonella* and *Shigella* spp.

A new method for testing phosphatase activity in bacteria, called MGP, has been described (28). This method proves as reliable as conventional ones and is easier than most of the tests commonly used in clinical microbiology as regards both technical execution and interpretation of results. In the MGP method, phosphatase activity is tested by inoculating microorganisms on agar plates containing the phosphate substrate phenolphthalein diphosphate (PDP) and the stain methyl green (MG) and observing colonies after 24 h of incubation. Colonies of phosphatase-positive strains, and only these, appear brightly green stained, whereas the area around them is completely unstained.

Since the MGP method was originally developed for gram-positive bacteria and failed to work satisfactorily with gram-negative strains (28), we decided to design an improved version of the method capable of detecting phosphatase activity in gram-negative bacteria as well.

In this paper, we describe this new version of the MGP method, and after extensive evaluation of phosphatase activity in *Enterobacteriaceae* using several different methods, we show that production of phosphatase is a constant property of all strains in the major species of *Enterobacteriaceae*.

MATERIALS AND METHODS

Strains and growth media. A total of 1,055 strains belonging to 65 different taxa of *Enterobacteriaceae* were analyzed in this study. Most strains were freshly isolated from specimens of various human origins, examined by standard procedures (17), and identified by the API 20E system (API Systems S.A., La Balme-les-Grottes, Montalieu-Vercieu, France). MacConkey agar (Difco Laboratories, Detroit, Mich.) was routinely used for colony isolation and strain maintenance.

As regards some of the most recently recognized species of *Enterobacteriaceae* which are not identified by the API 20E system, we tested reference strains obtained from either the American Type Culture Collection (ATCC) or the Collection of the Institut Pasteur (CIP). Reference strains were also used for a number of rare species which we never isolated in our laboratory although they could be identified by the API 20E system. All of these strains are listed in Table 4 along with the respective catalog numbers.

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Determination of phosphatase activities on bacterial strains. The phosphatase activities of the isolates were tested both by conventional methods and by variants of the MGP method. We used three different conventional methods, namely, the test proposed by Baird-Parker (2), the method of Barber and Kuper (3), and the method described by Bobey and Ederer (5). All of the tests were performed as recommended by the respective authors. The MGP method was performed as follows. Tryptose phosphate agar (Difco) was added with 50 μg of MG (Carlo Erba, Milan, Italy) per ml and 500 μg of a phosphate substrate per ml. Double-strength agar was used to prevent swarming of *Proteus* strains. Both dye and substrate from master solutions filtered through 0.22-μm-pore-size filters were added to tryptose phosphate agar which had been melted and kept at 44°C. Three different phosphate substrates, namely, PDP, phenolphthalein monophosphate (PMP), and 6-benzoylnaphthyl phosphate (6-BNP), all from Sigma Chemical Co., St. Louis, Mo., were in turn combined with the dye as described above. Test media (final pH, 7.3) were then poured into Petri dishes (20 ml per dish) and allowed to solidify. Isolated colonies of strains to be tested were then picked up with a needle and deposited on the agar surface. Colonies showing any degree of green staining after incubation at 37°C for 24 h were recorded as phosphatase positive.

Effects of phosphates on phosphatase activity. The effects of phosphates on phosphatase activity and colony staining were investigated in various batches of tryptose agar (Difco), each containing different concentrations of sodium phosphate, obtained by mixing together suitable amounts of monobasic and dibasic salts so as to maintain a constant pH of 7.3. MG and PDP, PMP, or 6-BNP were added after melting, and the melted media were poured into Petri dishes.

RESULTS

Effects of various phosphatase substrates on the staining of colonies formed by gram-negative strains in the presence of MG. In a previous work (29), we noticed that addition of purified phosphatase to a test tube containing MG plus either PMP or 6-BNP caused the formation of stained precipitates which were far more plentiful than those formed when PDP was the substrate. On the basis of this observation and given the assumption that in the MGP method staining of bacterial colonies was due to precipitation of complexes between phosphatase reaction products and the stain (29), we considered the possibility that substitution of substrates such as PMP and 6-BNP for PDP might increase the sensitivity of the MGP method.

All of the isolates formed unstained colonies in the presence of PDP but formed green-stained colonies on plates containing either PMP or 6-BNP and proved phosphatase positive in the conventional tests (Table 1). Ten *Staphylococcus aureus* isolates formed stained colonies with all of the substrates (including PDP) and proved phosphatase positive in the conventional tests.

Effect of phosphatase repression by phosphates on green staining of colonies of *Enterobacteriaceae* and staining of staphylococcal colonies in the presence of PMP and 6-BNP. To confirm that the green pigmentation of the colonies observed in the presence of PMP and 6-BNP was due to bacterial phosphatase activity, we evaluated the staining of the colonies by the MGP method and at increasing concentrations of phosphates in several strains of *Escherichia coli*, *Enterobacter aerogenes*, and *Serratia marcescens*, since phosphatase activity in these species of *Enterobacteriaceae* is known to be repressed by phosphates (4, 6, 34). All of the strains of *Enterobacteriaceae* were phosphatase negative with the conventional tests and formed unstained colonies by the MGP method when grown in the presence of 0.5 M phosphate, but they showed a progressive increase in both phosphatase activity and pigmentation of the colonies when grown in the presence of progressively lower phosphate concentrations (Table 2).

TABLE 1. Effects of various phosphate substrates on staining of colonies of gram-negative and gram-positive bacteria by the MGP method

Organism (no. of strains)[a]	Colony staining by the MGP method with the following substrate[b]		
	PDP	PMP	6-BNP
Escherichia coli (7)	−	+	++
Klebsiella pneumoniae (4)	−	+	++
Serratia marcescens (4)	−	+	++
Proteus vulgaris (4)	−	+	++
Staphylococcus aureus (10)	+	++	+++

[a] All of the strains were phosphatase positive by conventional tests.
[b] Symbols: −, absence of colony pigmentation; +, ++, and +++, increasing intensities of pigmentation (green, deep green, and very deep green, respectively).

It is also evident from Table 2 that all of the phosphatase-positive staphylococci formed green-stained colonies, whereas all of the phosphatase-negative strains formed unstained colonies, whatever the substrate used.

Phosphatase activities of various species of *Enterobacteriaceae* by three different conventional methods and various versions of the MGP method. All strains of virtually all of the species tested were phosphatase positive by all of the three different conventional methods and formed green-stained colonies with the MGP method when 6-BNP was used as the substrate. The only exception was the *Leminorella richardii* type strain, which was phosphatase positive but formed unstained colonies. In the *Edwardsiella tarda* type strain, in some strains of *Hafnia alvei*, and in all strains of the tribe *Proteeae*, the colonies were surrounded by a green-stained halo (Fig. 1).

With PMP as the substrate, the positivity was restricted to a few species and generally to a few strains within each of them (Tables 3 and 4). However, it is worth mentioning that all of the strains of *Proteeae* were phosphatase-positive with this substrate, and that some of their colonies (scattered among the various species of the tribe) were surrounded by a green halo (Fig. 1).

Finally, some strains of *Proteeae* and the *Rahnella aquatilis* type strain were the only strains of *Enterobacteriaceae* to form stained colonies even when PDP was the substrate.

DISCUSSION

The major aims of this work were (i) to modify the MGP method, which had proved to be both simple and accurate in detecting phosphatase activity of gram-positive bacteria (28), extending its use to gram-negative bacteria, and (ii) to perform an accurate evaluation of phosphatase activity in one of the most important bacterial groups for human disease, namely, the *Enterobacteriaceae*, so as to clarify the conflicting results described in the literature (9, 26, 33, 35).

Our data show that aim i was achieved by substituting 6-BNP for PDP. Under these conditions, all of the strains of *Enterobacteriaceae* which were phosphatase positive by conventional methods formed green-stained colonies,

TABLE 2. Phosphatase activity and colony staining of strains of *Enterobacteriaceae* and strains belonging to various staphylococcal species

Organism (no. of strains)	Phosphate concn (M)[a]	Phosphatase activity tested by conventional methods[b]			Colony pigmentation by the MGP method with the following phosphatase substrates[b]		
		p-NPP[c]	PDP-NH_3[d]	MUP[e]	PDP	PMP	6-BNP
Escherichia coli (10)	0	++	++	++	−	++	+++
	0.1	+/−	+/−	+/−	−	−	+
	0.5	−	−	−	−	−	−
Enterobacter aerogenes (10)	0	++	++	++	−	++	+++
	0.1	+/−	+/−	+/−	−	−	+
	0.5	−	−	−	−	−	−
Serratia marcescens (10)	0	++	++	++	−	++	+++
	0.1	+/−	+/−	+/−	−	−	+
	0.5	−	−	−	−	−	−
Staphylococcus aureus (18)	0.02	+	+	+	++	+++	+++
Staphylococcus epidermidis (11)	0.02	+	+	+	++	+++	+++
Staphylococcus hominis (9)	0.02	−	−	−	−	−	−
Staphylococcus saprophyticus (7)	0.02	−	−	−	−	−	−

[a] For staphylococci, the phosphate concentration corresponds to the disodium phosphate concentration of tryptose phosphate agar (2.5 g/liter).
[b] Symbols: −, absence of colony pigmentation; +, ++, and +++, increasing intensities of pigmentation (green, deep green, and very deep green, respectively).
[c] Described by Baird-Parker (2). p-NPP, *p*-Nitrophenyl phosphate.
[d] Described by Barber and Kuper (3).
[e] Described by Bobey and Ederer (5). MUP, 4-Methylumbelliferyl phosphate.

whereas both phosphatase-negative staphylococci and *E. coli* strains whose phosphatase activity had been repressed by P_i formed unstained colonies.

As far as aim ii is concerned, our findings show that production of phosphatase is a constant property of all strains of virtually all of the species of *Enterobacteriaceae* that occur in clinical specimens. These findings are apparently in contrast to those previously reported by some researchers. Conflicting results might depend on differences in the methods used, which may modify the sensitivity of the assay, as well as on the fact that different growth media, most likely containing different amounts of phosphates, were used. Such factors could easily lead to substantial variability of results, since phosphatase production is known to be regulated by phosphates in *E. coli* and in other species of the *Enterobacteriaceae* family (4, 6, 30, 34) and since substrate selectivity in the action of phosphatases has been reported (22).

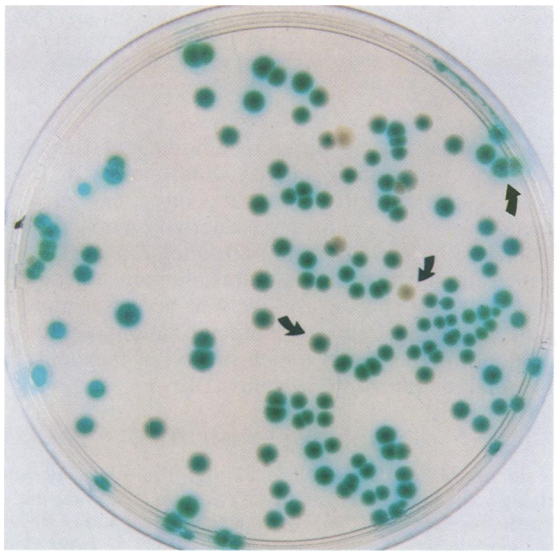

FIG. 1. Colony staining by the MGP method. A petri dish of tryptose phosphate agar medium containing MG and PMP was inoculated with a *Salmonella paratyphi* A (unstained colonies), a *Serratia marcescens* (stained colonies without halo), and a *Proteus vulgaris* (stained colonies with light halos) strain.

A pitfall common to most of the aforementioned works was the limited number of species of *Enterobacteriaceae* considered or the very few strains tested for each species or both factors. Moreover, since those studies were performed there have been radical changes in both the nomenclature and the classification of members of the *Enterobacteriaceae* family, which now contains as many as 24 genera subdivided into more than 100 taxa (8, 13–15). This paper considers 1,055 strains belonging to all of the 20 genera whose strains occur in clinical specimens (13) and to 65 of the 76 species (or biogroups or enteric groups) that these genera include.

The fact that *Enterobacteriaceae* are all invariably phosphatase positive could be very useful both for taxonomic purposes and in clinical laboratory practice, since it provides a simple and reliable way of separating the strains of this group from other gram-negative bacteria that grow in the selective media currently used for *Enterobacteriaceae* isolation. It is worth mentioning that strains of *Acinetobacter calcoaceticus*, *Alcaligenes* (*Achromobacter*) *xylosoxidans*, *Alcaligenes faecalis*, *Pseudomonas aeruginosa*, and other *Pseudomonadaceae*, which are MacConkey positive and glucose nonfermenting (27) and have been described as phosphatase negative (23, 24), all failed to produce stained colonies by the MGP method (data not shown).

In view of this, the property, typical of the MGP method, of revealing phosphatase activity by staining of the colonies but not the area around them appears quite useful. It should enable us to recognize single phosphatase-positive colonies

TABLE 3. Phosphatase activities of various strains of *Enterobacteriaceae* isolated in our laboratory as detected by the MGP method with different substrates

Organism (no. of strains)[a]	Colony pigmentation by the MGP method with the following phosphate substrate[b]:		
	PDP	PMP	6-BNP
Citrobacter amalonaticus (3)	−	−	+
Citrobacter diversus (4)	−	−	+
Citrobacter freundii (33)	−	−	+
Enterobacter aerogenes (40)	−	+/−	+
Enterobacter agglomerans (2)	−	−	+
Enterobacter cloacae (65)	−	+/−	+
Escherichia coli (326)	−	−	+
Hafnia alvei (19)	−	+/−	+h
Klebsiella oxytoca (28)	−	+/−	+
Klebsiella pneumoniae (142)	−	+/−	+
Klebsiella rhinoscleromatis (6)	−	−	+
Morganella morganii (29)	+/−	+h	+h
Proteus mirabilis (61)	−	+	+h
Proteus vulgaris (29)	+/−	+h	+h
Providencia alcalifaciens (5)	+/−	+h	+h
Providencia rettgeri (10	+/−	+h	+h
Providencia stuartii (15)	+/−	+h	+h
Salmonella anatum (6)	−	+	+
Salmonella derby (3)	−	+	+
Salmonella panama (8)	−	−	+
Salmonella paratyphi A (2)	−	−	+
Salmonella paratyphi B (2)	−	+	+
Salmonella typhi (39)	−	+	+
Salmonella typhimurium (8)	−	+	+
Salmonella wien (5)	−	−	+
Serratia liquefaciens (46)	−	+/−	+
Serratia marcescens (47)	−	+/−	+
Shigella flexneri (12)	−	+/−	+
Tatumella ptyseos (2)	−	+/−	+
Yersinia enterocolitica (16)	−	−	+
Yersinia frederiksenii (3)	−	+	+
Yersinia intermedia (2)	−	−	+
Yersinia kristensenii (3)	−	−	+
Yersinia pseudotubercolosis (2)	−	−	+

[a] The phosphatase activities of all of the strains were also evaluated by three different conventional methods, namely, those described by Baird-Parker (2), Barber and Kuper (3), and Bobey and Ederer (5). All strains of all species gave positive results with all three of the methods.

[b] Symbols: +, positive phosphatase reaction or green staining of colonies in all strains; +/−, green staining of colonies in a variable percentage of strains; −, complete absence of green staining of colonies; h, presence of stained halos around some colonies.

TABLE 4. Phosphatase activities of reference strains of rare species of *Enterobacteriaceae* as detected by the MGP method with different substrates

Strain[a]	Colony pigmentation by the MGP method with the following phosphatase substrate[b]:		
	PDP	PMP	6-BNP
Cedecea davisae CIP 8034	−	−	+
Cedecea lapagei CIP 8035	−	−	+
Cedecea neteri ATCC 35855	−	+	+
Edwardsiella tarda CIP 7861	−	+	+h
Enterobacter amnigenus ATCC 33072	−	−	+
Enterobacter sakazakii ATCC 29544	−	−	+
Escherichia fergusonii ATCC 35469	−	−	+
Escherichia hermanii ATCC 33650	−	−	+
Escherichia vulneris ATCC 29943	−	−	+
Ewingella americana CIP 8194	−	−	+
Klebsiella planticola CIP 8136	−	−	+
Klebsiella terrigena CIP 8007	−	−	+
Kluyvera ascorbata CIP 7953	−	−	+
Kluyvera cryocrescens CIP 8001	−	−	+
Koserella trabulsi ATCC 35313	−	−	+
Leminorella grimontii ATCC 33999	−	−	+
Leminorella richardii ATCC 33998	−	−	−
Proteus penneri ATCC 33519	+	+h	+h
Providencia rustigianii ATCC 33673	−	+h	+h
Rahnella aquatilis CIP 7865	+	+	+
Salmonella subgenus II CIP 8229	−	−	+
Salmonella subgenus IIIa CIP 8230	−	−	+
Salmonella subgenus IIIb CIP 8231	−	−	+
Salmonella subgenus IV CIP 8232	−	−	+
Salmonella subgenus V CIP 8233	−	−	+
Serratia ficaria CIP 7923	−	−	+
Serratia fonticola CIP 7864	−	−	+
Serratia odorifera CIP 7901	−	−	+
Serratia plymuthica CIP 7712	−	+	+
Shigella boydii CIP 5248	−	+	+
Shigella sonnei CIP 8249	−	−	+

[a] The phosphatase activities of all of the strains were also evaluated by three different conventional methods, namely, those described by Baird-Parker (2), Barber and Kuper (3), and Bobey and Ederer (5). All strains of all of the species gave positive results with all three of the methods.

[b] Symbols: +, positive phosphatase reaction or green staining of colonies in all strains; +/−, green staining of colonies in a variable percentage of strains; −, complete absence of green staining of colonies; h, presence of stained halos around some colonies.

easily on plates crowded with phosphatase-negative colonies and vice versa (Fig. 1).

Phosphatase genes have been inserted in some cloning vehicles to produce fusion proteins synthesized by bacteria for export. In these systems, the clones into which DNA fragments have been inserted are recognized as colonies that have lost phosphatase activity (32). Substituting this new version of the MGP test for conventional methods will afford the possibility of identifying a single phosphatase-negative colony in a plate overcrowded with thousands of phosphatase-positive colonies, thus allowing the phosphatase gene to be exploited for selection of fusion genes at least as easily as with the β-galactosidase method, with the important advantage that the MGP test is 10 to 100 times cheaper.

Another characteristic of the MGP method is that it can be prepared in various versions with different sensitivities. In a forthcoming paper (R. Pompei et al., manuscript in preparation), we intend to show that this particular property of the method can be exploited for separating phosphatase-positive species which produce different amounts of the enzyme. Preliminary results indicate that some strains of the tribe *Proteeae*, especially those from the genus *Providencia*, seem to be endowed with a phosphatase activity much higher than that of all other strains of *Enterobacteriaceae*. Both the incidence and the distribution of this phenomenon among the various *Proteeae* species are likely to be better understood after accurate biochemical characterization of the strains that belong to this tribe, which are often misidentified by commercial identification systems (10).

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